

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand on NK Cells Protects From Hepatic Ischemia-Reperfusion Injury

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Background. Ischemia-reperfusion injury (IRI) significantly contributes to graft dysfunction after liver transplantation. Natural killer (NK) cells are crucial innate effector cells in the liver and express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a potent inducer of hepatocyte cell death. Here, we investigated if TRAIL expression on NK cells contributes to hepatic IRI.

Methods. The outcome after partial hepatic IRI was assessed in TRAIL-null mice and contrasted to C57BL/6J wild-type mice and after NK cell adoptive transfer in RAG2/common gamma-null mice that lack T, B, and NK cells. Liver IRI was assessed by histological analysis, alanine aminotransferase, hepatic neutrophil activation by myeloperoxidase activity, and cytokine secretion at specific time points. NK cell cytotoxicity and differentiation were assessed in vivo and in vitro.

Results. Twenty-four hours after reperfusion, TRAIL-null mice exhibited significantly higher serum transaminases, histological signs of necrosis, neutrophil infiltration, and serum levels of interleukin-6 compared to wild-type animals. Adoptive transfer of TRAIL-null NK cells into immunodeficient RAG2/common gamma-null mice was associated with significantly elevated liver damage compared to transfer of wild-type NK cells. In TRAIL-null mice, NK cells exhibit higher cytotoxicity and decreased differentiation compared to wild-type mice. In vitro, cytotoxicity against YAC-1 and secretion of interferon gamma by TRAIL-null NK cells were significantly increased compared to wild-type controls.

Conclusions. These experiments reveal that expression of TRAIL on NK cells is protective in a murine model of hepatic IRI through modulation of NK cell cytotoxicity and NK cell differentiation.

Keywords: NK cells, Tumor necrosis factor-related apoptosis-inducing ligand, Hepatic ischemia-reperfusion injury, NK cell maturation, Cytotoxicity.

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Hepatic ischemia-reperfusion injury (IRI) is characterized by parenchymal inflammation associated with apoptosis and necrosis of hepatocytes and sinusoidal endothelial cells, thereby leading to liver graft dysfunction (1, 2). This process

is modulated by paracrine interactions with circulating non-parenchymal inflammatory cells, including Kupffer cells, dendritic cells, T cells, and natural killer (NK) cells (3). In the human liver, NK cells are the main innate lymphoid cell population and interact with non-parenchymal and parenchymal cells through activating and inhibitory receptors, thereby shaping innate immune responses through the main

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effector functions that consist of cytotoxicity and cytokine secretion (4–7).

Tumor necrosis factor apoptosis-inducing ligand (TRAIL, Apo2L) is a type II membrane bound TNF family ligand, which was originally identified on the basis of sequence homology to Fas ligand (FasL) and tumor necrosis factor (TNF) (8, 9). Although TRAIL is constitutively expressed on mRNA level in a wide variety of normal tissues, the expression of functional TRAIL protein appears to be rather restricted to immune cells, including NK cells, T cells, monocytes, dendritic cells, and neutrophils (10–16). Interestingly, hepatic NK cells express TRAIL whereas mature splenic NK cells are TRAIL negative (17). Furthermore, the expression of TRAIL on hepatic NK cells seems to critically modulate NK cell-induced hepatocyte cell death (18).

In this study, we explored the role of TRAIL on NK cells in a murine model of partial hepatic IRI. We show an up-regulation of TRAIL post-IRI and a significant elevation

of liver injury after warm liver ischemia in TRAIL-null mice compared to wild-type mice. In addition, we observed increased recruitment of granulocytes into the liver in TRAIL-null mice in response to IRI and elevated cytotoxicity of NK cells from TRAIL-null mice in vivo and in vitro. The importance of TRAIL expression on NK cells during IRI was confirmed by adoptive transfer experiments of wild-type and mutant TRAIL-null NK cells into RAG2/common gamma-null mice that lack B, T, and NK cells. These results support a novel role of TRAIL and NK cells in the regulation of liver damage induced by IRI.

RESULTS

Deletion of TRAIL is Associated With Increased Hepatic IRI

Hepatic injury in response to IR was assessed in TRAIL-null and wild-type mice. All animals survived the

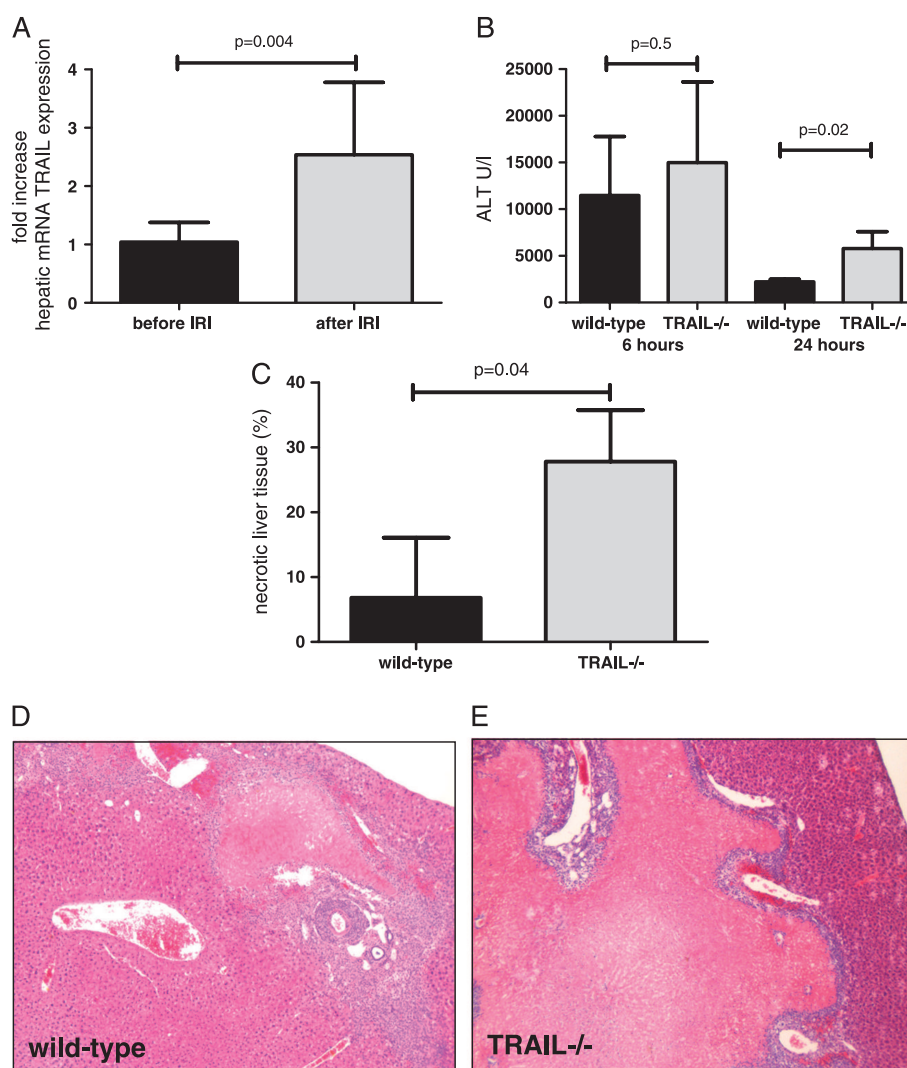


FIGURE 1. Liver injury and degree of liver necrosis is increased in TRAIL-null compared to wild-type mice after partial hepatic IRI. In wild-type mice, hepatic expression of TRAIL was significantly increased at 24 hr of partial hepatic IRI compared to untreated animals (A). Assessment of liver injury by serum ALT levels 24 hr after hepatic IRI showed significantly higher levels in TRAIL-null mice compared to wild-type mice (B). Four days after IRI, the extent of necrotic tissue in TRAIL-null mice was significantly higher compared to wild-type mice (C). Representative images of livers with HE staining at $\times 50$ magnification (wild-type mice in D, TRAIL-null mice in E). Mean values \pm SD are shown, Mann-Whitney test, $n=10$ animals per group.

postoperative course until harvest at indicated time points. IRI resulted in a significant increase of TRAIL mRNA expression in the livers of wild-type animals compared non-injured controls (Fig. 1A). Liver injury was assessed by alanine aminotransferase (ALT) measurements and increased 6 hr of reperfusion in wild-type and TRAIL-null animals (Fig. 1B). ALT levels declined after 24 hr in both groups; however, they were significantly higher in TRAIL-null compared to wild-type mice (Fig. 1B). TRAIL-null mice showed significantly higher histological signs of necrotic liver tissue compared to wild-type mice 4 days after reperfusion (Fig. 1C–E).

Next, we examined the extent of neutrophil activation and infiltration by MPO activity and Gr-1 positive immunoreactivity. There was significantly higher MPO activity in the livers of TRAIL-null compared to wild-type mice (Fig. 2A). Correspondingly, there was a significantly higher number of

Gr-1 positive cells in TRAIL-null mice 24 hr after reperfusion as assessed by immunohistochemistry (Fig. 2B–F). TRAIL-null mice showed a significant increase of the pro-inflammatory acute phase cytokine IL-6 3 hr after partial hepatic ischemia (Fig. 3A), whereas TNF alpha (Fig. 3B), IL-1 beta (Fig. 3C), and IL-5 (Fig. 3D) did not differ significantly between the groups.

TRAIL Deficiency in NK Cells is Associated With Increased Liver Injury and Oxidative Burst After Partial Hepatic Ischemia

Next, we questioned the impact of NK cells and more specifically TRAIL expression on NK cells during hepatic IRI. Adoptive transfer of TRAIL-null and wild-type NK cells into RAG2/common gamma-null recipients was performed. Adoptive transfer of TRAIL-null NK cells was associated with significantly elevated liver injury compared to wild-type NK cells

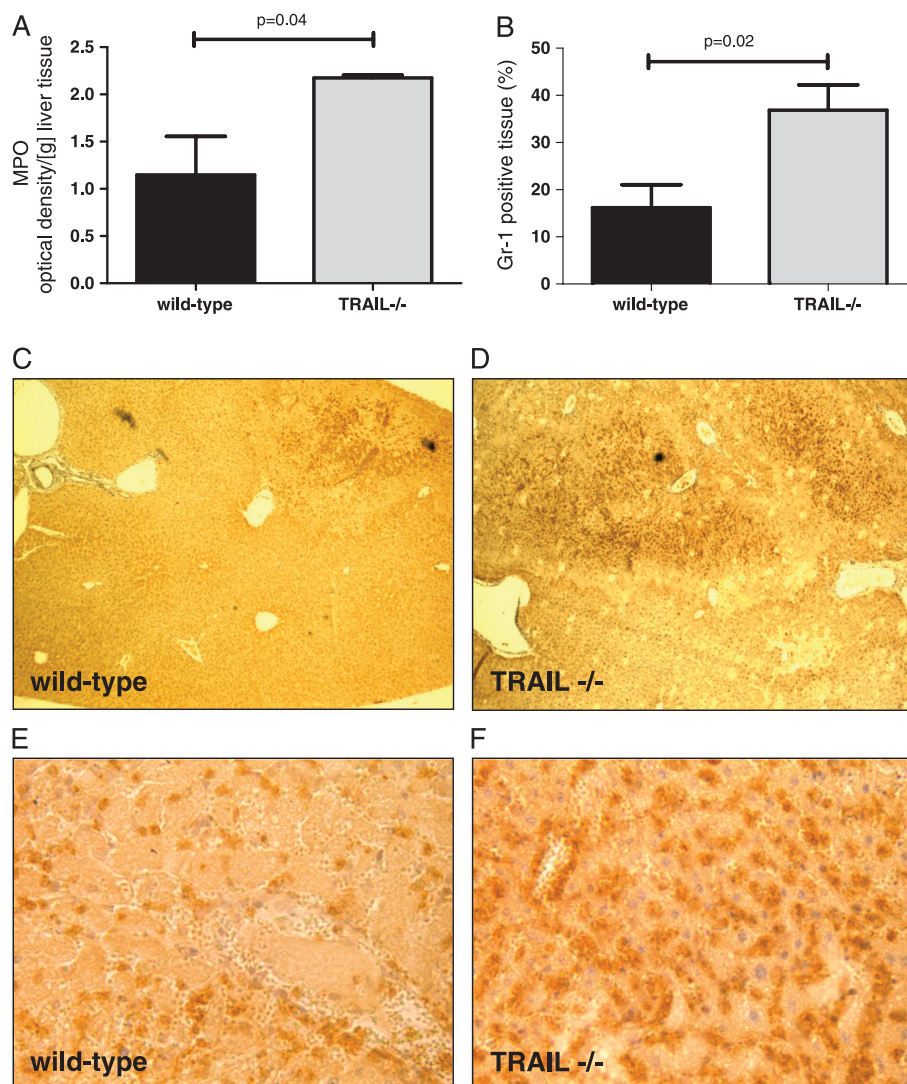


FIGURE 2. Neutrophil infiltration and activation is increased in TRAIL-null compared to wild-type mice after partial hepatic IRI. Myeloperoxidase activity in the liver was significantly higher in TRAIL-null mice compared to wild-type mice after hepatic IRI (A). Twenty-four hours after IRI, a significantly higher influx of Gr-1 positive cells was detectable in livers from TRAIL-null mice compared to wild-type mice (B, mean values±SD, Mann-Whitney test, n=8 animals per group). Representative images of livers with immunostaining for Gr-1 at ×50 (C, D) and ×400 (E, F) magnification (wild-type in C and E, TRAIL-null in D and F).

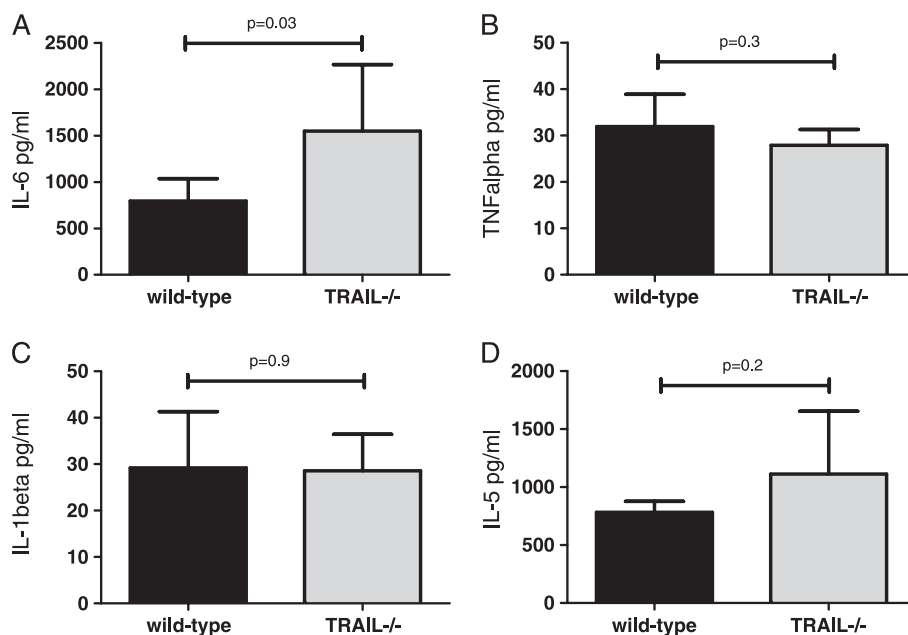


FIGURE 3. Increased serum levels of IL-6 in TRAIL-null mice. Serum cytokine levels were measured after 3 hr of reperfusion in TRAIL-null and wild-type mice. IL-6 levels were significantly increased in TRAIL-null mice compared to wild-type mice (A), whereas TNF alpha, IL-1 beta, and IL-5 were not significantly different between the groups (B–D). Mean values \pm SD, Mann-Whitney test, $n=8$ animals per group.

(Fig. 4A). Furthermore, MPO levels and expression of Gr-1 positive cells in the ischemic liver lobe were significantly increased after transfer of TRAIL-null NK cells compared to wild-type NK cells (Fig. 4B and C).

Cytotoxicity is Increased in NK Cells in TRAIL-Null Mice After IRI

Cytotoxicity and cytokine secretion of NK cells and depend on the state of differentiation (5, 19). Expression of the cytotoxic granule protein CD107a (LAMP-1) on the cell surface is a marker of lysosomal degranulation and cytotoxicity in NK cells. CD107a positive NK cells were significantly increased in non-ischemic liver lobes in mice null for TRAIL compared to wild-type mice (Fig. 5A). After partial hepatic IRI, cytotoxic CD107a positive NK cells were further increased in TRAIL-null livers compared to wild-type livers (Fig. 5A). NK cell cytotoxicity in vitro was assessed using NK cell-sensitive YAC-1 target cells. Sorted TRAIL-null NK (NK1.1 positive, CD3 negative) cells showed significantly elevated cytotoxicity compared to wild-type NK cells (Fig. 5B).

Decreased Levels of Immature NK Cells and Increased Secretion of Interferon Gamma Secretion by TRAIL-Null NK Cells

The surface marker CD27, a member of the TNF-receptor superfamily, allows to distinguish mature (CD27 low) from immature (CD27 high) NK cells in mice. Fractions of mature (CD27 low) NK cells were significantly decreased in livers of TRAIL-null mice compared to wild-type mice under basal conditions and after hepatic IRI (Fig. 5C and D). Further analysis of other subsets of lymphocytes revealed no differences in CD3 positive lymphocytes and fractions of NKT (Figure S1, SDC, <http://links.lww.com/TP/A964>). Interferon gamma secretion from sorted NK cells in vitro

was significantly higher in TRAIL-null NK cells compared to wild-type NK cells in response to IL-12 and IL-18 stimulation (Fig. 5E).

DISCUSSION

This study investigated the role of TRAIL on NK cells during partial hepatic IRI. Hepatic injury was significantly increased in TRAIL-null mice in comparison to wild-type mice. Adoptive transfer of TRAIL-null NK cells into RAG2/common gamma-null mice was also associated with increased liver injury, emphasizing the importance of TRAIL on NK cells in tissue protection during IRI.

Warm ischemia of the liver leads to damage of hepatocytes and subsequent increase of serum ALT levels in response to release of oxygen-free radicals, cytokines, and chemokines, which then activate cascades of the pro-inflammatory immune response (20). Interestingly, parameters of tissue damage and inflammation were elevated in TRAIL-null mice. In response to these processes, the acute phase cytokine IL-6 that is secreted from parenchymal and non-parenchymal hepatic cells during liver injury (21–23) again was increased in TRAIL-null mice. Interestingly, hepatic TRAIL expression in wild-type mice was per se increased after IRI compared to untreated mice. This fact indicates an up-regulation of hepatic TRAIL expression during liver ischemia and reperfusion and might constrain further liver damage by clearing, for example, apoptotic and necrotic cells and probably blocking NK cells activation as seen in TRAIL-null mice.

NK cells are cytotoxic lymphocytes of the innate immune system and can recognize stressed cells in the absence of antibodies or major histocompatibility complex (MHC) class 1 (24). In our study, TRAIL deficiency on NK cells was associated with elevated cytotoxicity after NK-cell stimulation in vitro and in vivo. Previous studies revealed that

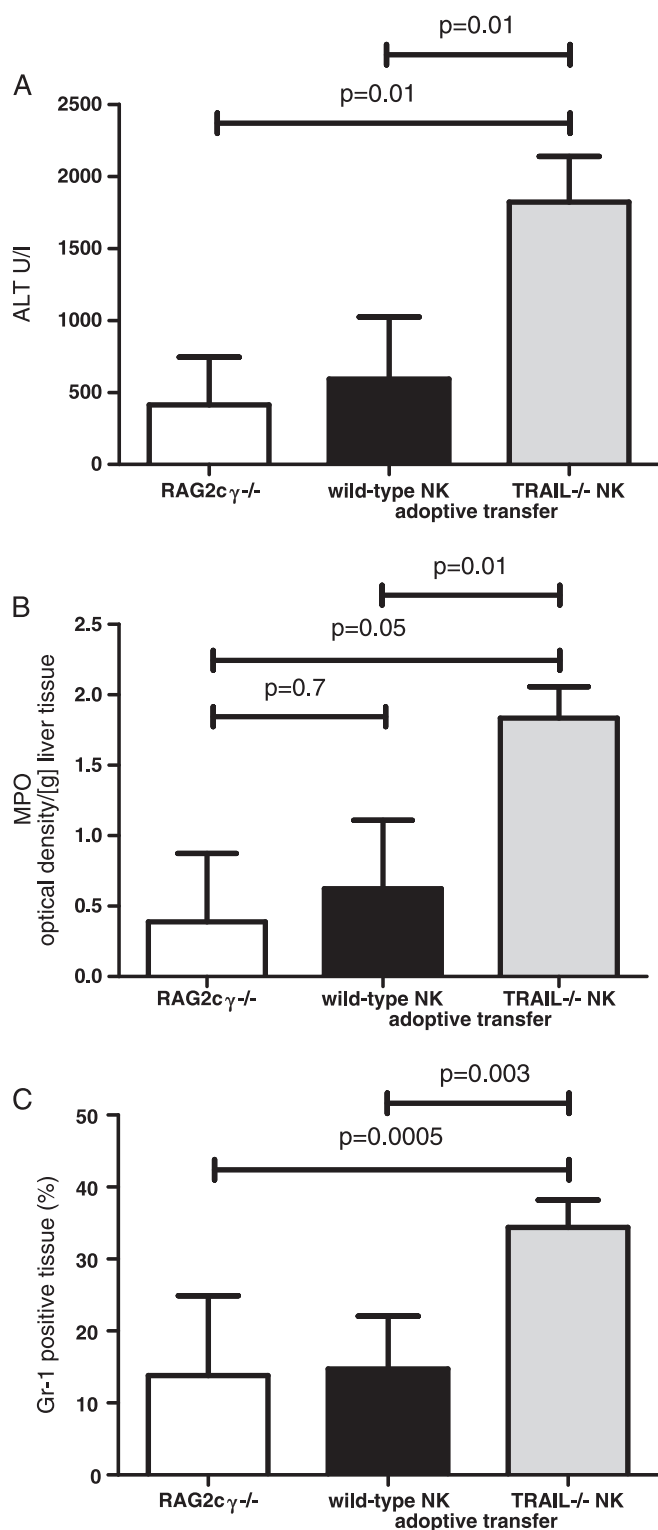


FIGURE 4. TRAIL expression on NK cells mediates liver injury. Adoptive transfer of wild-type and TRAIL-null NK cells was performed into Rag2/common gamma-null mice that lack B, T, and NK cells. Liver injury, myeloperoxidase activity, and Gr-1 positive cells in liver section were assessed 24 hr after hepatic IRI. Liver injury was significantly increased after adoptive transfer of TRAIL-null NK cells compared to wild-type NK cells (A). Myeloperoxidase activity in the liver and influx of Gr-1 positive cells was significantly higher after adoptive transfer of TRAIL-null NK cells compared to wild-type NK cell adoptive transfer (B, C). Mean values \pm SD, Mann-Whitney test, n=6–8 animals per group.

injury and repair is modulated by NK cells via lysis of stressed or infected cells, thereby modulating injury and repair (25). We show that NK cell-dependent exocytosis of cytotoxic granules occurs on the cell surface through the exposure of the endosomal membrane protein CD107a (LAMP-1) (16, 26).

There is evidence that NK cells promote hepatic IRI in a phase-specific pattern (27, 28). As shown in our study, TRAIL seems not just to impact on cytotoxicity but also on secretion of interferon gamma by NK cells. Previously, we have shown that dysfunctional NK cell-dependent secretion of interferon gamma is associated reduced liver injury, revealing the relevance of NK cells on cellular crosstalk during IRI (29). Hepatic IRI is associated with increased levels of interferons (29–32). However, the secretion interferon gamma is rather associated with a local acute phase reaction and is not sufficient to maintain hepatic IRI alone (33). Local alterations of interferon gamma seem to be sufficient to explain the observed phenotype as systemic levels of interferon gamma were too low for detection. Elevated secretion of interferon gamma as observed in our experiments is a potential forward feedback for the expression of TRAIL on NK cells as shown in other models (14).

The lower frequency of mature NK cells in TRAIL-null mice potentially results from decreased activation and mobilization of hepatic NK cells. TRAIL expression on NK and other cells such as dendritic or Kupffer cells potentially modulates NK cell mobilization via auto- and paracrine interactions in response to an inflammatory stimulus and presence of danger associated molecular patterns (DAMPs) as described in other models (34). Previous studies revealed that an excessive immune response may be limited through TRAIL and required direct interactions with resident or circulating antigen presenting cells (35, 36). TRAIL expression on innate lymphoid cells has been shown to be protective in IRI but is associated with elevated injury in a model of concanavalin A-mediated liver damage (37). However, in hepatic IRI mainly paracrine interactions and DAMPs activate NK cell activation, whereas the lectin concanavalin A mainly activates NKT cells rather than NK cells and thereby contributes to hepatocyte apoptosis in a different manner. These results are supported by recent findings in which we showed that interactions of NK cells with Kupffer cells are protective in a model of partial hepatectomy (19).

In conclusion, these experiments demonstrate that the expression of TRAIL on NK cells is required to limit liver injury in a model of hepatic IRI. TRAIL controls hepatic NK-cell cytotoxicity, cytokine secretion, and the recruitment of NK cells to the liver with consecutive reduced influx of granulocytes and hepatocellular necrosis and injury.

MATERIALS AND METHODS

All research protocols were carried out in accordance with the National Institutes of Health guidelines for the care and use of experimental animals and approval of the Animal Care Committee of the Canton Bern, Switzerland.

Animal Preparation and Experimental Setting

Experiments were performed using mice on the C57BL/6J background. Wild-type C57BL/6J and TRAIL-null (TRAIL^{-/-}) mice were bred and maintained in the Central Animal Facility of the University of Bern, Switzerland (38). Rag2/common gamma-null (RAG2c γ ^{-/-}) mice were purchased from Taconic (Germantown, NY). Animals were housed with a 12-hr light-dark

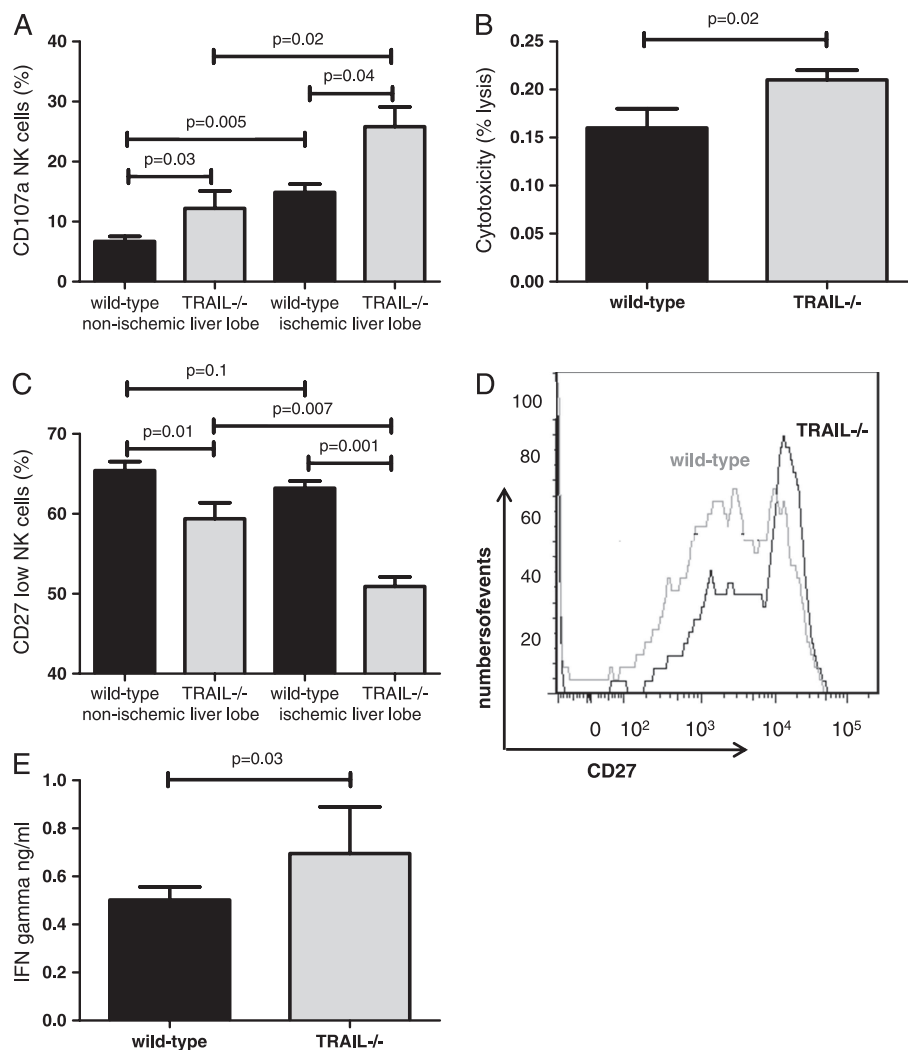


FIGURE 5. Deletion of TRAIL on NK cells impacts on effector function and maturation of NK cells in vivo and in vitro. Three hours after hepatic IRI, the percentage of cytotoxic CD107a positive cells was significantly increased in TRAIL-null mice compared to wild-type mice (A). In vitro, cytotoxicity of sorted TRAIL-null NK cells against YAC-1 cells was significantly increased compared to wild-type NK cells (B). The fraction of mature CD27 low in non-ischemic liver lobes of TRAIL-null mice was reduced compared to wild-type mice (C). In addition, the decrease of CD27 low NK cells after induction of partial hepatic ischemia was higher in TRAIL-deficient mice compared to wild-type mice, suggesting a change of NK-cell mobilization during hepatic ischemia (C, D). Secretion of interferon gamma in response to stimulation with IL-12 and IL-18 was significantly higher in TRAIL-null NK cells compared to wild-type NK cells (E). Mean values \pm SD, Mann-Whitney test, $n=8$ animals per group.

cycle at 22°C. All operations were performed in animals at the age of 8 to 12 weeks on a warming plate to maintain body temperature. The abdominal contents were rinsed during the operation with saline to avoid drying-out. Buprenorphin (Reckitt Benckiser AG, Switzerland) was administered subcutaneously after 6 hr for postoperative analgesia and in further post-operative course depending on the general condition of the animals. Blood samples were taken after 3 and 6 hr of reperfusion. At specific time points, mice were anesthetized, blood was taken from the inferior vena cava, and liver lobes were removed and further processed.

Surgical Procedures

Under general anesthesia with xylazine 10 mg/mL (Graeb AG, Switzerland), ketamine 80 mg/kg (Pfizer AG, Switzerland), and heparin 200 U/kg (Bichsel AG, Switzerland), an oblique laparotomy was performed and the hepatic artery and portal vein of the left liver lobe were temporarily clamped for 60 min under control of body temperature. Hepatic veins were not clamped. After removal of the clamp, the abdomen was closed in two layers and buprenorphin was administered subcutaneously for analgesia.

Rag2/common gamma-null mice (Taconic, Denmark) were used for adoptive transfer experiments by injecting a total of 10^6 sorted splenic NK (NK1.1 positive, CD49b positive, CD3 negative) cells in 100 μ L of phosphate buffered saline (Invitrogen Life Technologies, Darmstadt, Germany) into the saphenous vein under intraperitoneal general anesthesia using medetomidine 0.5 mg/kg (Graeb AG, Switzerland), clonazepam 5 mg/kg (Graeb AG, Switzerland), and fentanyl 0.05 mg/kg (Janssen-Cilag, Switzerland). Surgery was performed 6 days after adoptive transfer as described above.

Immunohistochemical Analysis of the Liver

Fresh liver tissue was fixed overnight in 4% paraformaldehyde. For hematoxylin-eosin (HE) staining, sections were deparaffinized with xylol and then counterstained with HE. The degree of liver necrosis was assessed in HE-stained liver tissue (four high-power fields, 40 times magnification) after 4 days of liver reperfusion using a computerized program (ImageJ). For granulocyte staining, slides of paraformaldehyde-fixed tissue were dewaxed, rehydrated, and blocked by endogenous peroxidase 3% H_2O_2 . After antigen retrieval, first anti-Gr-1 antibody (Gr-1 anti-mouse Ly-6G,

eBioscience, #4-5931-85) and second biotinylated anti-rat antibody (#E0468 Dako, Denmark) were applied. After incubation of the tissue sections with DAB, slides were counterstained with hematoxylin (H-51275; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Finally, Gr-1 positive and negative areas were measured in each animal on four high-power fields of liver tissue with 40 times magnification 24 hr after liver reperfusion using a computerized program (ImageJ).

Measurement of Liver Injury

ALT levels in the serum were measured 6 and 24 hr after liver reperfusion by a photometric UV test measuring the oxidation of NADH to NAD (Modular Analytics EVO, P800; Roche, Germany).

Measurements of Cytokine Levels by Enzyme-Linked Immunosorbent Assay

The serum concentrations of tumor necrosis factor (TNF) alpha, interleukin (IL)-6, IL-1 beta, and IL-5 were examined after 3 hr of liver reperfusion by enzyme-linked immunosorbent assay using immunoassay kits (PeproTech Inc., Rocky Hill, NJ) according to the recommended protocol.

Hepatic Neutrophil Detection by Myeloperoxidase Assay

Myeloperoxidase assay was carried out as previously described (39). Briefly, liver samples were thawed, 50 mg of tissue was weighed out and homogenized in 1 mL of 20 mM phosphate buffer using a TissueLyser (Qiagen) 2 min at 20 Hz, centrifuged at 10,000g for 10 min, and the pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide. The samples were then freeze-thawed four times using liquid nitrogen followed by a 40-sec sonication, centrifuged at 10,000g for 10 min, and the supernatant was used for the MPO assay. The assay mixture contained 10 μ L of the sample, 25 μ L 3,3',5,5'-tetramethylbenzidine (final concentration 1.6 mM), 25 μ L H₂O₂ in 80 mM phosphate buffer (pH 5.4, final concentration H₂O₂ 0.3 mM), and 40 μ L of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide. The mixture was incubated at 37°C for 2 min, stopped with the addition of 1 M HCl, and the absorbance was measured at 450 nm.

Quantitative TaqMan PCR

RNA was isolated from snap-frozen ischemic liver samples by Trizol Reagent according to the manufacturer's protocol (Life Technologies). cDNA was synthesized by using Omniscript RT kit 200 (Cat. No. 205113; Qiagen). mRNA of TRAIL in ischemic livers was analyzed by qRT-PCR (ABI 7900, SDS 2.3 software), and primers and probes sequences were ready to use from Applied Biosystems (Tnfsf10, #Mm00437174_m1, Rotkreuz, Switzerland). As reference gene control (RG), beta actin (#Mm00607939_s1; Rotkreuz, Switzerland) was used. The relative changes in mRNA were calculated with the $\Delta\Delta C_t$ method, whereas C_t values of target gene expression (TG) were calculated relative to the RG using the following formula: $\Delta C_{tTG} = C_{tTG} - C_{tRG}$. Experimental groups (TG) were normalized to control group (CG): $\Delta\Delta C_t = \Delta C_{tTG} - \Delta C_{tCG}$, fold increase = $2^{-\Delta\Delta C_t}$.

Antibodies

The following antibodies were used for flow cytometry analysis: anti-mouse NK1.1 (APC), CD107a (PE), CD3 (FITC), CD4 (PE), CD27 (PE-Cy7), CD49b (PE-Cy7), CD8 (PE), and CD19 (PE), which were purchased from eBioscience. A SORP LSRII cytometer (BD Bioscience) was used for flow cytometry analysis, and data were analyzed using FlowJo software. A FACSARIA III cell sorter (BD Bioscience) was used for NK cell-sorting experiments.

Purification of Liver Mononuclear Cells and Splenic NK Cells

Ischemic and non-ischemic liver lobes were excised during harvest and passed through a 200G stainless steel mesh. After centrifugation of the filtrate at 50g for 3 min, the supernatant was collected, the non-parenchymal cell supernatant fraction was washed once and resuspended

in a 35% Percoll (GE Healthcare) solution, and overlaid on a 70% Percoll solution. After cell separation at 800g for 20 min, the interphase was collected and the cells were stained with specific antibodies. Finally, cell analysis was performed by flow cytometry. Spleens were excised and NK cells were purified to perform adoptive transfer experiments, cytotoxicity, and interferon gamma secretion assays. Depletion of CD4, CD8, and CD19 (all PE-labeled) positive cells was performed by using electromagnetic beads following the manufacturer's protocol (Miltenyi Biotec Inc., Auburn, CA). The flow-through was labeled with NK1.1 (APC), CD49b (PECy7), and CD3 (FITC) for sorting of NK1.1/CD49b positive CD3 negative NK cells on a FACSARIA III.

Cytotoxicity Assay

Sorted primary murine NK cells were used for a 4-hr ⁵¹Cr-release assay using the NK cell-sensitive murine lymphoma cell line YAC-1 as target cells. YAC-1 cells were radiolabeled with 500 mCi of ⁵¹Cr (PerkinElmer, NEZ03000) per 500 μ L cell suspension for 1 hr at 37°C. After washing, YAC-1 cells were incubated for 1 hr at 37°C and then washed again. Isolated primary murine NK cells were sorted by flow cytometry as described above. NK cells and YAC-1 cells were mixed at a ratio of 1:20 in a U-bottom 96-well plate. After incubation for 4 hr at 37°C and 5% CO₂/air atmosphere, 10% Triton X-100 was added to each positive control. The release of ⁵¹Cr was measured in the supernatant using a gamma counter (COBRA II; Packard S/N 404470) and the specific lysis was calculated.

Interferon Gamma Secretion Assay of NK Cells

Isolated and sorted splenic NK cells were plated in a 96-well plate. After incubation with IL-12 and IL-18 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) overnight, interferon gamma secretion was measured in the supernatant using an immunoassay kit (PeproTech) according to the recommended protocol.

Statistics

All data are expressed as geometric means \pm standard deviations. For statistical analysis, a nonparametric test (Mann-Whitney test) was used. Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA). Data with *P* less than 0.05 was considered as statistically significant.

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